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Cadmium-binding polypeptides in microalgal strains with laboratory-induced cadmium tolerance

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ABSTRACT: Strains of 5 algal species that have been induced to develop tolerance to previously toxic cadmium (Cd) concentrations were investigated for production of the Cd-binding polypeptides, poly(γ-glutamylcysteinyl)glycines, $(\gamma\text{-EC})_n$G. Liquid chromatography using Sephadex G-50, ICP analysis of Cd in chromatographic fractions, and amino acid analysis provided clear evidence of $(\gamma\text{-EC})_n$G production in the 2 most tolerant species, *Phaeodactylum tricornutum* and *Dunaliella tertiolecta*. Production of Cd-binding peptide in *P. tricornutum* was positively correlated with cellular Cd content, on a dry-weight basis. In contrast, Cd-tolerant strains of *Isochrysis galbana, Pavlova lutheri,* and *Tetraselmis maculata* did not produce detectable $(\gamma\text{-EC})_n$G. Although Cd-tolerant *I. galbana* differed from the control strain in some aspects of polypeptide content, as seen by the production of thiol-rich compounds, the specific mechanism for enhanced Cd tolerance in the 3 species that do not produce $(\gamma\text{-EC})_n$G remains unknown.

INTRODUCTION

Cadmium (Cd) has been identified as a 'priority pollutant' by the U.S. Environmental Protection Agency (US EPA 1979, Eisler 1985), at least in part because elevated concentrations have been found in fishery products intended for human consumption (Hall et al. 1976). This demonstrates that Cd does accumulate in marine biomass, despite the limited solubility of free Cd^{2+} in seawater (Eaton 1976, Sunda et al. 1978). One possible route by which Cd may enter the marine food web is through uptake by phytoplankton exposed to sub-lethal concentrations of this ion. The inhibitory effect of Cd upon microalgae has been documented in culture experiments (Jennings 1979, Rachlin et al. 1982, 1983, Wikfors & Ukeles 1982, Fisher et al. 1984, Peterson & Healey 1985) and studies of natural phytoplankton assemblages (Wolter et al. 1984, Frithsen et al. 1987). Interspecific differences in microalgal Cd tolerance were shown. Moreover, exceptionally tolerant strains of several algal species have been isolated from contaminated environments (Stokes et al. 1977, Murphy et al. 1982, Bariaud & Maestre 1984) or selected experimentally in the laboratory (Li 1980, Wikfors & Ukeles 1982, Collard & Matagne 1990).

Understanding of mechanisms by which some algae tolerate Cd has progressed only recently with the recognition of 'non-metallothionein binding proteins' (Stone & Overnell 1985). Cysteine-rich polypeptides that form aggregates in the presence of Cd were first identified in yeast cells (Murasugi et al. 1981, Kondo et al. 1985) and later in higher plants (Grill et al. 1985, Robinson et al. 1987). Several names have been given to this class of molecules; they are most precisely called poly(γ-glutamylcysteinyl)glycines, $(\gamma\text{-EC})_n$G, based upon the structure of repeating γ-glutamylcysteine dipeptides with a single terminal glycine (Robinson & Jackson 1986). In plants, $(\gamma\text{-EC})_n$G have been shown conclusively to bind Cd and to be induced by exposure to this metal ion (Robinson et al. 1988, Delhaize et al. 1989). Involvement of $(\gamma\text{-EC})_n$G in the mechanism of metal tolerance has also been demonstrated (Delhaize et al. 1989).

Evidence that microalgal Cd tolerance is achieved by a mechanism which includes binding of induced polypeptides to this metal ion was first obtained with *Euglena gracilis* (Gingrich et al. 1984, Weber et al. 1987, 1988). Recent research (reviewed by Robinson 1989) has shown that heavy metal-binding polypeptides produced by algal species from several classes are

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Table 1. Algal strains used in experiments

Species	Strain designation	Medium	Conc. Cd (μ M)
Isochrysis galbana	*I. galbana*	ASP_2[a]	
	I. galbana Cd 2.5	ASP_2 + 2.5 mg% $CdCl_2$	136
Pavlova lutheri	*P. lutheri*	ASP_2	
	P. lutheri Cd 2.5	ASP_2 + 2.5 mg% $CdCl_2$	136
Phaeodactylum tricornutum	*P. tricornutum*	ASP_2	
	P. tricornutum Cd 5	ASP_2 + 5 mg% $CdCl_2$	272
	P. tricornutum Cd 10	ASP_2 + 10 mg% $CdCl_2$	544
	P. tricornutum Cd 20	ASP_2 + 20 mg% $CdCl_2$	1088
Dunaliella tertiolecta	*D. tertiolecta*	ASP_2	
	D. tertiolecta Cd 10	ASP_2 + 10 mg% $CdCl_2$	544
Tetraselmis maculata	*T. maculata*	ASP_2	
	T. maculata Cd 5	ASP_2 + 5 mg% $CdCl_2$	272

[a] Concentrations of trace metals and chelators (l^{-1}) in the standard medium formulation are as follows: $FeCl_3 \cdot 6H_2O$, 0.829 mg; $ZnCl_2$, 0.44 μg; $MnCl_2 \cdot 4H_2O$, 1.25 mg; $CoCl_2 \cdot 6H_2O$, 3.1 μg; $CuCl_2$, 1.18 μg; $H_3BO_3 \cdot 6.16$ mg; Na_2EDTA, 30 mg; tris(hydroxymethyl)aminomethane, 1 g

of the $(\gamma\text{-EC})_nG$ type; synthesis of $(\gamma\text{-EC})_nG$ is induced by Zn, Pb, Ag, Cu, and Hg in addition to Cd (Gekeler et al. 1988, Maita & Kawaguchi 1989). In the present study, we looked for $(\gamma\text{-EC})_nG$ in strains of 5 algal species that have been selected, through gradual concentration increases over several years in culture, for expression of tolerance to Cd concentrations that are lethal upon initial exposure of the parent strain (Wikfors & Ukeles 1982).

MATERIALS AND METHODS

Algal culture and extraction. The following microalgal species were used (Table 1): *Isochrysis galbana* Parke, *Pavlova lutheri* (Droop) Green, *Phaeodactylum tricornutum* Bohlin, *Dunaliella tertiolecta* Lerche, and *Tetraselmis maculata* Butcher. Stock strains of these species have been maintained for many years in artificial seawater medium ASP_2 (Provasoli et al. 1957). Previously, Cd-tolerant sub-strains were developed from the above isolates through slow adaptation in progressively higher Cd concentrations (Wikfors & Ukeles 1982). Strain designations for the Cd tolerant strains reflect the Cd concentration, in mg% $CdCl_2$, to which the strain has been adapted for over 10 yr. Cd molar concentrations are listed in Table 1.

Algae were cultured in 500 ml Erlenmeyer and 2800 ml Fernbach flasks containing 250 and 1200 ml of medium, respectively. Flask cultures were incubated at 20 °C with illumination at 300 μE m^{-2} s^{-1} from cool-white fluorescent bulbs on a 12/12 h light/dark cycle. Cells were harvested for analysis after 28 d of growth. Previous experience had shown that these strains were in a nitrogen-deficient stationary phase at this time. Evidence for this lies in the incorporation of all medium nitrogen into algal protein before Day 28, even though rates of growth were limited by another factor, perhaps carbon, after 14 to 21 d (Wikfors & Ukeles 1982, Wikfors et al. unpubl.). Algal cells were removed from culture media and rinsed with isotonic NaCl by cold (3 °C) centrifugation at 1020 × *g* for 15 min. The pellet of algal cells was then resuspended in 10 ml of a buffered solution (10 mM Tris-HCl, pH 7.4, 10 mM KCl, 1.5 mM $MgCl_2$, 50 mM 2-mercaptoethanol). A small volume of culture was retained for cell counting in an Improved Neubauer hemocytometer (Bright Line)*. Cells were homogenized with an ultrasonic cell disrupter, and insoluble material was removed by cold centrifugation at 1500 × *g* for 30 min. The supernatant was poured into polypropylene tubes and shipped on wet ice from Milford in Los Alamos.

Separation of Cd-binding components of cell extracts by gel-filtration. At Los Alamos, algal extracts were centrifuged at 15 000 × *g* for 10 min to remove insoluble material. The supernatant was collected and passed through a 2.5 × 100 cm column containing Sephadex G-50 (fine) (LKB Biotechnology, Inc., Piscataway, NJ, USA) equilibrated against a solution containing 50 mM Tris-HCl, pH 7.8. Fractions (8 ml) were collected and analyzed for thiol content. Thiol-rich fractions were further analyzed for Cd content.

Detection of thiol-rich compounds. Thiol-rich compounds were detected by the method of Ellman (1959).

* Mention of trade names does not imply endorsement

The solution to be tested was mixed with an equal volume of a solution containing 0.075 mM dithionitrobenzoic acid and 50 mM KH_2PO_4 titrated to pH 7.6 with NaOH. Samples were allowed to develop for 5 min, and then the OD_{405} was measured with a Perkin Elmer, Model 553 UV/VIS Spectrophotometer.

Detection of Cd. Cd was detected with a Perkin-Elmer Model 5500 Inductively Coupled Plasma (ICP) Spectrophotometer calibrated with Cd in 50 mM Tris-HCl, pH 8.0.

Purification of thiol-rich compounds by gel affinity chromatography. Fractions from the Sephadex column which contained both thiols and Cd were pooled and the volume reduced by ultrafiltration in an Amicon Model 202 ultrafiltration unit containing a YC05 filter (500 Da, molecular-weight exclusion filter, Amicon Corp., Danvers, MA, USA) under nitrogen. The concentrated samples were then loaded onto a cold (4 °C) 1 ml thiopropyl Sepharose column (Sigma Chemical Co., St. Louis) equilibrated against a solution containing 50 mM Tris-HCl, pH 7.8 (initial flow rate, 50 ml^{-1} h). Metal-binding polypeptides were retained by the column and Cd was released. The column was then washed first with 50 ml of a solution containing 50 mM Tris-HCl, pH 8.0, 1 M NaCl, then with 50 ml of 50 mM Tris-HCl, pH 8.0. Polypeptides were eluted from the column at a flow rate of 3 ml^{-1} h with 50 mM 2-mercaptoethanol in 50 mM Tris-HCl, pH 8.0. Samples were measured for thiol content after repeated dilution and concentration by ultrafiltration through a YC05 filter to remove 2-mercaptoethanol. Fractions containing thiol-rich molecules were pooled and their volume again reduced by ultrafiltration.

Determination of amino acid content of metal-binding complexes. Concentrations of thiol-rich compounds were determined by Ellman assay (Ellman 1959) using reduced glutathione as the standard. Aliquots containing 1 to 5 µg of polypeptide were dried in an acid-cleaned test tube. HCl (200 µl) containing 0.5 % (v/v) phenol was added to the bottom of a vacuum vial. The tube containing the polypeptides was placed in the vacuum vial and the vial was sealed under vacuum after being flushed with 3 alternate vacuum-nitrogen cycles. The polypeptides were hydrolyzed for 24 h at 116 °C. The vial was then cooled, excess HCl was removed with tissue, and the sample was dried under vacuum. Twenty µl of derivatization reagent [7 : 1 : 1 : 1 (v/v) ethanol : triethylamine (TEA) : water : phenylisothiocyanate (PITC)] was then added to the redried sample. Samples were capped and allowed to stand at 22 °C for 20 min before being placed under vacuum for 1 h to remove the PITC and dry the sample. The dried sample was redissolved in 20 µl methanol. HPLC analysis of hydrolyzed samples was accomplished by separation on a 15 cm × 3.9 mm Pico-Tag amino acid analysis column (Waters Associates, Milford, MA, USA). Eluent A contained 0.14 M sodium acetate, 0.5 ml TEA, pH 6.4; Eluent B contained 60 % acetonitrile in water. The linear gradient was 10 % B to 53 % B in 10 min. Flow rate was 1.0 ml min^{-1}. Amino acids were detected with a UV detector set at 254 nm. The column was calibrated previously with a standard amino acid mix. The process was found to underestimate cysteine in the samples. Therefore, results were compared to those obtained for glutathione, $(\gamma\text{-EC})_1G$, and a correction factor for the amount of cysteine present was calculated, based upon values obtained for the amino acid content of this compound.

RESULTS

Initial screening for Cd-binding polypeptides was conducted with 250 ml cultures of 5 Cd-tolerant algal strains. Final population densities and numbers of cells extracted are given in Table 2. In 2 algal strains, *Phaeodactylum tricornutum* Cd 20 and *Dunaliella tertiolecta* Cd 10, a thiol-rich peak similar in size to metal-binding complexes containing $(\gamma\text{-EC})_nG$ in higher plants was considered the first presumptive evidence of

Table 2. Screening of Cd-tolerant algal strains for metal-binding polypeptides

Strain designation	Cells ml^{-1} ($\times 10^6$)	Extracted cells ($\times 10^6$)	Thiol peak[a]	Cd (ppm)[b]	Cu (ppm)	Fe (ppm)
I. galbana Cd 2.5	3.65	730	Broad, few thiols	1.67	0.10	0.01
P. lutheri Cd 2.5	2.34	468	Not present	0.70	0.10	0.02
P. tricornutum Cd 20	5.09	1020	Broad, thiol-rich	36.34	0.09	0.04
D. tertiolecta Cd 10	1.74	348	Broad, thiol-rich	10.64	0.05	0.02
T. maculata Cd 5	0.39	78	Not present	0.24	0.03	0.01
Extraction buffer[c]	–	–	–	0.19	0.01	<0.01

[a] Resolved on G-50 Sephadex column, in addition to 2-mercaptoethanol peak
[b] Analyzed by ICP of fractions normally containing $(\gamma\text{-EC})_n$Gs for Cd, Cu, and Fe, respectively
[c] Extraction buffer used for extraction of algae, containing 2-mercaptoethanol

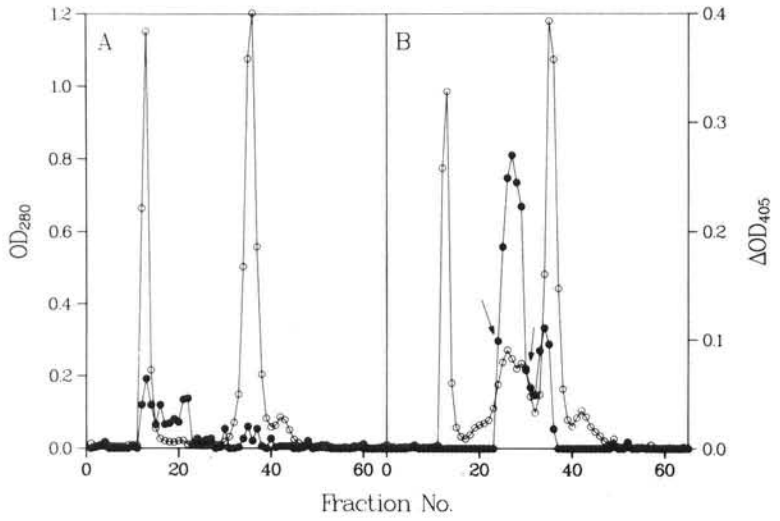

Fig. 1. Analysis of soluble portions of extracts from (A) *Isochrysis galbana* grown in media without additional Cd, and (B) *I. galbana* Cd 2.5 adapted to and cultured in 2.5 mg% $CdCl_2$. Separation was by passage through a Sephadex G-50 (fine) gel filtration column. OD_{280} (○——○) and change in absorbance at 405 nm after addition of Ellman's reagent (ΔOD_{405}; ●——●) were measured for each fraction. Arrows in (B) mark limits of fractions collected for futher analysis

metal-binding polypeptides in the samples. *Isochrysis galbana* Cd 2.5 yielded a broad peak containing very low thiol concentrations, and the remaining 3 strains showed no evidence of thiol-rich compounds in the same size range (2 to 4 kD) (Table 2). ICP analysis of fractions in which Cd-binding polypeptides would be expected, revealed elevated Cd concentrations in *P. tricornutum* Cd 20 and *D. tertiolecta* Cd 10; whereas assays for Cd gave uncertain results for *I. galbana* Cd 2.5. The remaining 3 strains showed no elevated Cd in the tested fractions. In no case was Cu or Fe elevated above background concentrations in fractions analyzed for these metal ions; this is the expected result because these metal ions are present in only traces in the growth medium (Table 2).

The presence of Cd-enriched thiol fractions in at least 2 algal strains provided the impetus for a more thorough analysis of these species to ascertain whether fractions did, in fact, contain $(\gamma\text{-EC})_n$G complexes. Fernbach-flask cultures provided sufficient biomass for these analyses. In Figs. 1 to 3, 2 measurements are plotted for Sephadex G-50 fractions of crude algal extracts: (1) optical density at 280 nm (OD_{280}) for constitutive proteins, nucleic acids and other absorbing materials (peaks centered at ca 800 and 6000 d), and (2) the change in absorbance at 405 nm before and after addition of Ellman's reagent (ΔOD_{405}). It is clear from the presence of thiols (the change in OD_{405}; Fig. 1) that tolerant *Isochrysis galbana* cells produced cysteine-rich complexes in the presence of Cd; control (Cd-free) cells produce no detectable cysteine-rich complexes. ICP analysis of pooled column fractions 25 to 30 from *I. galbana* Cd 2.5 extracts showed Cd concentrations almost 9-fold above background levels in these fractions. *Dunaliella tertiolecta* cultured in 10 mg% $CdCl_2$ yielded a thiol-rich peak smaller, relative to OD_{280} peaks, than *I. galbana,* and this peak was somewhat shifted to a higher apparent molecular weight. Fractions 16 to 24 (Fig. 2) were pooled for further analysis. Cd concentrations were 56-fold higher than background in these fractions. The most thorough analysis was conducted on *Phaeodactylum tricornutum* extracts because strains tolerant to 3 different concentrations of Cd were available; cells were also grown in media which did not contain this ion. Thiol-rich, ΔOD_{405} peaks were found in extracts from 3 of the 4 *P. tricornutum* strains. These peaks were centered uniformly at Fractions 27 to 28 (Fig. 3), and contained elevated Cd concentrations compared to background levels.

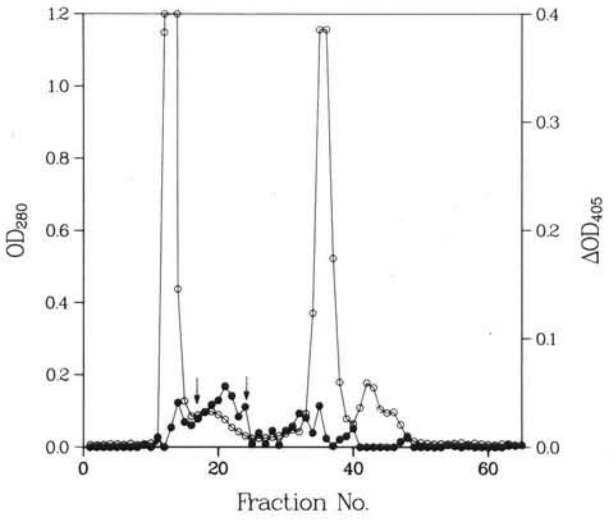

Fig. 2. Analysis of the soluble portion of an extract from *Dunaliella tertiolecta* (strain Cd 10) adapted to and cultured in 10 mg% $CdCl_2$. Separation was by passage of the extract through a Sephadex G-50 (fine) gel filtration column. Absorbance at 280 nm (OD280; ○——○) and change in absorbance at 405 nm after addition of Ellman's reagent (ΔOD_{405}; ●——●) were measured for each fraction. Arrows mark the limits of fractions collected for content analysis

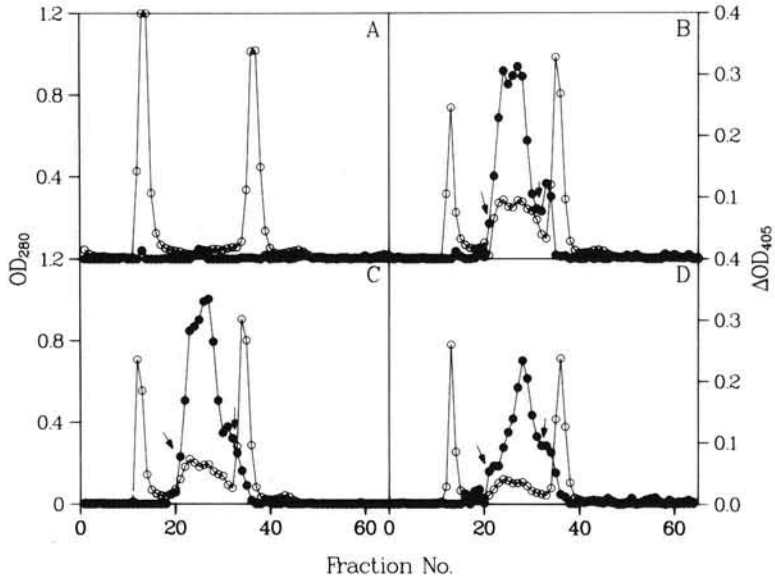

Fig. 3. Analysis of soluble portions of extracts from (A) *Phaaedactylum tricornutum* grown in media with no added Cd, and of *P. tricornutum* Cd 5, 10 and 20 growing in 5, 10 and 20 mg% $CdCl_2$ (B to D respectively). Separation was by passage through a Sephadex G-50 (fine) gel filtration column. OD_{280} (○——○) and change in absorbance at 405 nm after addition of Ellman's reagent (DOD_{405}; ●——●) were measured for each fraction. Arrows mark the limits of fractions collected for content analysis

When pooled fractions, indicated by arrows in Figs. 1 to 3, were subjected to amino acid analysis, ratios of glutamate : cysteine were not consistent with the presence of $(\gamma\text{-EC})_n$G in *Phaeodactylum tricornutum* Cd 5 or *Isochrysis galbana* Cd 2.5, and no other evidence suggested the presence of these compounds in any of these strains. However, glutamate : cysteine ratios in *P. tricornutum* Cd 10 and Cd 20, and *Dunaliella tertiolecta* Cd 10 suggested the presence of these metal-binding polypeptides in these cells (Table 3). Ratios of glutamate and cysteine to glycine indicate that *P. tricornutum* produces a mixture of $(\gamma\text{-EC})_2$G and $(\gamma\text{-EC})_3$G that is relatively rich in the latter peptide. In contrast, *D. tertiolecta* appears to produce a complex containing almost equal amounts of the 2 peptides (Table 3). Algal metal-binding complexes differ from those isolated from *D. innoxia* cell cultures included as a control; these extracts contain essentially all $(\gamma\text{-EC})_2$G (Table 3).

DISCUSSION

The present study confirms a report of $(\gamma\text{-EC})_n$G production in *Phaeodactylum tricornutum* grown in elevated Cd concentrations (Maita & Kawaguchi 1989). Isolation of these metal-binding polypeptides from *Dunaliella tertiolecta* extends the list of microalgal taxa producing this class of Cd-binding compounds to the Polyblepharidaceae; other Chlorophycean taxa also possess this capacity (Gekeler et al. 1988). Because these species do produce Cd-binding polypeptides, insensitivity of these species to Cd toxicity upon initial exposure, relative to other microalgal species, may be

Table 3. Amino acid analyses of algal and control samples: analyses conducted on thiol-rich fractions separated by gel filtration through a Sephadex G-50 column. Values in nmol per 50 µl

Sample	Glutamate	Cysteine	Cysteine (corrected[a])	Glycine	Ratio[b] Glu:Gys:Gly
P. tricornutum Cd 5	0.3049	0.2495	0.6487	0.2712	1.12:2.39:1
P. tricornutum Cd 10	2.8686	1.0897	2.8330	1.1054	2.83:2.78:1
P. tricornutum Cd 20	2.5962	1.0545	2.7417	0.9514	2.73:2.87:1
D. tertiolecta Cd 10	2.1888	0.9120	2.3712	0.9310	2.35:2.55:1
I. galbana Cd 2.5	0.2500	0.2546	0.6620	0.1297	1.93:5.12:1
D. innoxia[c]	1.2582	0.5093	1.3200	0.6873	1.83:1.92:1
Glutathione	1.8220	0.6996	1.8226	1.8430	0.99:0.99:1

[a] Preparation of the polypeptides and glutathione resulted in an under-representation of cysteine in the assay. The above values for glutathione, in which the ratio is known to be 1:1:1, show this. Corrected cysteine values were obtained by factoring in the 0.99:0.38 under-analysis indicated by the glutathione results
[b] Ratios were calculated with corrected cysteine values
[c] Cell cultures grown in 10 µM Fe and 250 µM Cd (Fraction 1)

Table 4. Relationship between cellular Cd concentration and poly(γ-glutamylcysteinyl)glycine concentration in cadmium-tolerant algal strains. Values in μg per 10^6 cells

Algal strain	Cd	$(\gamma\text{-EC})_n$G
P. tricornutum Cd 5	0.204	0.84
P. tricornutum Cd 10	0.558	5.86
P. tricornutum Cd 20	0.987	8.16
D. tertiolecta Cd 10	0.105	2.91
I. galbana Cd 2.5	0.096	Not present

attributed to a mechanism which includes the production of these polypeptides (Wikfors & Ukeles 1982). Moreover, long exposure of these species to inhibitory, but sub-lethal Cd concentrations, may have selected for enhanced $(\gamma\text{-EC})_n$G or glutathione biosynthesis, thereby permitting greater Cd tolerance. Robinson (1989) comments that this adaptive mechanism has not been demonstrated conclusively in the algae. However, he outlines 4 specific ways in which this may occur: '(1) Increased activity of enzymes involved in $(\gamma\text{-EC})_n$G biosynthesis, (2) increased activity of enzymes responsible for S^{2-} saturation of metal-$(\gamma\text{-EC})_n$G complexes, (3) modified compartmentation of one of the components, $(\gamma\text{-EC})_n$G, S^{2-}, or metal, (4) modified rates of $(\gamma\text{-EC})_n$G turnover.'

Evidence for Cd adaptation in *Phaeodactylum tricornutum* by selection for more robust activity of enzymes involved in $(\gamma\text{-EC})_n$G metabolism can be found in the present study. During experimental development of Cd-tolerant strains in this species, sub-cultures in intermediate Cd concentrations gave rise to progressively more tolerant populations (Wikfors & Ukeles 1982). *P. tricornutum* Cd 10 and Cd 20 strains grow in concentrations that are acutely toxic to the original *P. tricornutum* strain from which they were derived, and both of these tolerant strains were shown in the present study to produce much $(\gamma\text{-EC})_n$G. According to this scenario, *P. tricornutum* 'constitutively' possesses the capacity to produce $(\gamma\text{-EC})_n$G in response to elevated Cd, but this response is insufficient in speed, magnitude or duration to permit survival at Cd concentrations greater than about 5 mg% $CdCl_2$. Production of large amounts of Cd-binding polypeptide by strains adapted to higher Cd concentrations indicates that enzymic reactions must be somehow enhanced over levels achieved by non-adapted strains. Furthermore, the amount of Cd-binding polypeptide produced is approximately proportional to the concentration of metal tolerated – as well as being proportional to the weight-normalized Cd content of the cells (Table 4). This argues against mechanisms (3) and (4) of Robinson (1989). As enzyme rates were not measured in this study, the specific point in the biochemical pathway at which adaptation occurred, or whether or not tolerance is actually associated with the changes in the pathway, remains somewhat ambiguous, although part of the biochemical mechanism of Cd tolerance in this species is now known.

The likelihood that different algal species have different specific mechanisms for ameliorating toxic trace metal stress was also suggested by Robinson (1989). In *Dunaliella tertiolecta*, evidence is strong that Robinson's mechanism (3), modified compartmentation, is employed. In this case, perhaps extracellular dumping of the polypeptide-metal complex occurs. Dry weight-specific concentrations of both Cd and $(\gamma\text{-EC})_n$G in *D. tertiolecta* Cd 10 are much lower than for *Phaeodactylum tricornutum* cultured in the same medium (Table 4). Previous studies suggesting that *D. tertiolecta* tolerates metal toxicants through some exclusion mechanism (Davies 1976, Fisher et al. 1984) do not address the question of whether metal ions are 'detoxified' within or outside of the cell. Our finding that *D. tertiolecta* synthesizes polypeptides with high affinity for Cd upon exposure to this ion renders less attractive the hypothesis that this species tolerates elevated Cd by simply resisting uptake. The presence of $(\gamma\text{-EC})_n$G in medium that has supported population growth of *D. tertiolecta* Cd 10 will need to be established to confirm this hypothesis. More important, the question of the ultimate fate of polypeptide-metal complexes in plant cells (i.e. where do complexes go once formed) remains unanswered. Further work on *D. tertiolecta* may provide at least partial answers to some of these questions.

Perhaps more surprising than the identification of $(\gamma\text{-EC})_n$G in *Phaeodactylum tricornutum* and *Dunaliella tertiolecta* is the apparent lack of evidence for the presence of these molecules in Cd-adapted strains of the other 3 species. Previous studies have not found any examples of microalgal strains which do not produce these molecules under Cd stress, and this mechanism has been suggested as a generalized plant response (Gekeler et al. 1988). However, it has also been determined that the ability to produce large quantities of these metal-binding polypeptides does not, in itself, confer metal tolerance upon plants (Jackson et al. 1989). The production of these polypeptides represents only part of the tolerance mechanism. It should be noted that the absence of evidence for the presence of these polypeptides in several algal strains does not preclude the presence of these metal-binding compounds under other conditions. Nevertheless, the sensitivities of the 3 strains not showing $(\gamma\text{-EC})_n$G synthesis in our analyses, relative to *P. tricornutum* and *D. tertiolecta* both upon initial exposure (Wikfors & Ukeles 1982) and after over 10 yr of exposure, suggests that the mechanism which requires polypeptide synthesis may be disabled and/or supplanted by an alternate, less effective tolerance mechanism that limits Cd concentrations ultimately

tolerated by some microalgae. In *Isochrysis galbana* and *Pavlova lutheri*, cellular exclusion of the metal apparently is not an effective tolerance mechanism because these species accumulate considerably more Cd on a dry weight-normalized basis than *D. tertiolecta* or *P. tricornutum* (Wikfors et al. unpubl.).

Isochrysis galbana is particularly intriguing. It is clear (Fig. 1) that extracts from the Cd-tolerant strain differ in cysteine content, compared to the control culture, although the ratio of glutamate : cysteine appears to preclude $(\gamma\text{-EC})_n$G biosynthesis. Has it another cysteine-rich metal-binding polypeptide? Li (1980) found evidence of an inducible cytosolic moiety of ca 16 kD that accumulated Cd as *I. galbana* cells grew in medium containing this ion, although immediate Cd binding appeared to occur in fractions with a molecular weight < 3 kD. The cysteine-rich peak found in the current study may correspond to the latter fractions from Li's work. Furthermore, if our Cd-tolerant strain responded similarly to Li's, then the bulk of the Cd taken up by the cells would eventually be passed to a larger 16 kD complex. To extend this hypothesis, our thiol-rich fractions and Li's 3 kD material may both represent accumulation of certain amino acids as precursors or degradation products of unidentified metal-binding polypeptides. High cellular uptake of Cd by *I. galbana* that cannot be accounted for by $(\gamma\text{-EC})_n$G binding is consistent with the findings of Li (1980). In the absence of alternate hypotheses, it is possible that *Pavlova lutheri* and *Tetraselmis maculata*, the other 2 species that showed no evidence of $(\gamma\text{-EC})_n$G biosynthesis may also develop Cd tolerance through production of a similar, alternate metal-binding compound. Facts arguing against such a unifying hypothesis are the lack of thiol-rich fractions from Sephadex columns of extracts from the latter 2 species, and differences between species in dry weight-specific Cd uptake. Nevertheless, the lack of evidence for $(\gamma\text{-EC})_n$G production in *I. galbana*, *P. lutheri*, and *T. maculata*, coupled with evidence of acquired Cd tolerance in these species, suggests that other adaptive mechanisms may occur in some microalgae to ameliorate Cd stress.

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